International Doctoral School
Medical University of Lodz

MEDICAL UNIVERSITY OF LODZ

DEVELOPMENT OF ADVANCED MEDICINAL THERAPY PRODUCTS (ATMPS) WITH PARTICULAR REFERENCE TO UNIVERSAL (FOR EACH RECIPIENT) CAR-T (T LYMPHOCYTES WITH A CHIMERIC ANTIGEN RECEPTOR) AND CAR-M (MACROPHAGES WITH A CHIMERIC ANTIGEN RECEPTOR)

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Introduction

This research focused on the development and implementation of an *in vitro* genetic modification system to silence the *B2M* gene encoding the β2-microglobulin protein, a key component of the MHC class I involved in allogeneic cells transplant rejection [1]. The T cell receptor (TCR) α chain-encoding gene, *TRAC*, was identified as an ideal target for genetic manipulation to disrupt TCR formation and reduce T cells alloreactivity [2] to overcome the issue with transplant rejection and GVHD.

Another advantage of this approach is the use of induced pluripotent stem cells (iPSCs) as a source of universal cells. They can self-renewal and differantiate into any mature cells from the three germ layers, including T lymphocites and macrophages [3]. Universal macrophages with CAR molecules will be obtained to address the challenge of heterogeneity of tumor-associated antigens (TAAs) in solid tumors [4].

In this study the use of CRISPR-Cas9 gene editing tool will be used to knock-out (KO) of the *B2M* (T cells and macrophages) and *TRAC* (T cells) genes in iPSCs, which further will be differentiated into T cell and macrophades (Fig.1)

Materials and methods

The first step was to **differentiate normal iPSCs** into induced neural stem cells (**Figure 2**), which were subsequently genetically modified **to knock out the *B2M* gene** using CRISPR/Cas9 lentiviral vectors-based tools.

To obtain **lentiviral vectors**, plasmids encoding specific **sgRNA sequences** were amplified in competent bacteria by transformation and cultured under selective conditions. Plasmid DNA was then isolated from bacteria, and after qualitative and quantitative assessment, used in the process of obtaining lentivirus particles. Next, iNS parental cells were **transduced with lentiviral vectors**. Briefly, the cells were exposed to viral particles and then cultured under selective conditions. After the selection process, the cells were cultured under standard conditions for their propagation and analysis.

To verify the knock-out of the *B2M* gene, **Western Blot** (at the protein level) and **Real-time PCR** (at the mRNA level) techniques were used, and the results are presented in **Figure 3**.

Results

The differentiation of iPS cell into iNS cell has been confirmed by anaylysing the expression of lineage-specific markers SOX2 (greensignal) and Nestin (redsignal) (**Figure2.**).

The results of Western Blot analysis indicated the loss of band intensity in the region of the reference band of 10 kDa (where β-2-microglobulin is a protein with a mass of 11 kDa) following transduction of iNS parental cells with vectors encoding sgRNA sequence 2 and a combination of all three sgRNAs., with the latter showing complete suppression of the *B2M* gene, resulting in a lack of beta-2-microglobulin protein even after treatment with IFN-γ. In iNS ko B2M gRNA2 cells, after the addition of IFN-γ, we observed a band corresponding to a mass of 11 kDa (**Figure 3A**). The Real-time PCR analysis, showed differences in the level of B2M expression in each cell line. iNSC parental cells were chosen as the positive control. The level of expression in transduced cells relative to parental cells decreased approximately 4.5-fold for sgRNA1 and 1.7-fold for sgRNA3, with this differences not being statistically significant **(Figure 3B**).

We did not observe complete suppression of the B2M gene in cells transduced with only one vector (single sgRNA). These findings may suggest that the analyzed population was heterogeneous and only a portion of cells underwent *B2M* knock-out or that only one allele of *B2M* has been silenced.

Conclusions

The results demonstrate the mastery of genetic engineering techniques and the introduction of genome editing elements to silence the *B2M g*ene in iPS cells-derived iNSC. Preliminary analyses have shown that silencing expression is possible using different sgRNAs with varying efficiency, with the highest efficiency achieved in iNS parental cells transduced with a combination of all three sgRNAs.

Further analyses are necessary, including the selection and propagation of clones with confirmed B2M knock-out, followed by reprogramming to obtain induced pluripotent stem cells and differentiation into macrophages while simultaneously silencing the TRAC gene and reprogramming and differentiating into T lymphocytes.

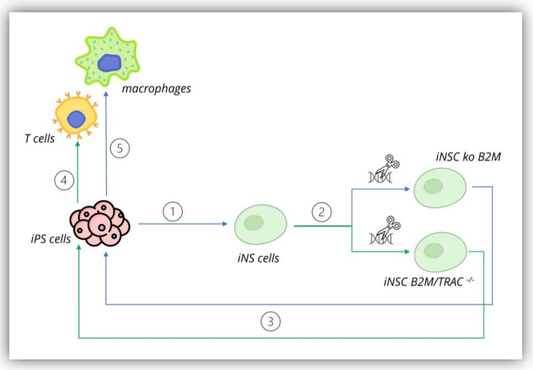

Figure 1. Diagram summarizing the stages of the research project. 1 - differentiation of normal iPS cells into neural stem cells (iNSCs); 2 - genetic modification using CRISPR-Cas9 technique, targeting the B2M (for macrophages) and B2M and TRAC genes (for T lymphocytes); 3 - reprogramming of iNSCs to iPSCs; 4 - differentiation of iPS ko B2M/TRAC cells cells into T lymphocytes; 5 - differentiation of iPS ko B2M cells into macrophages. Source: own work.

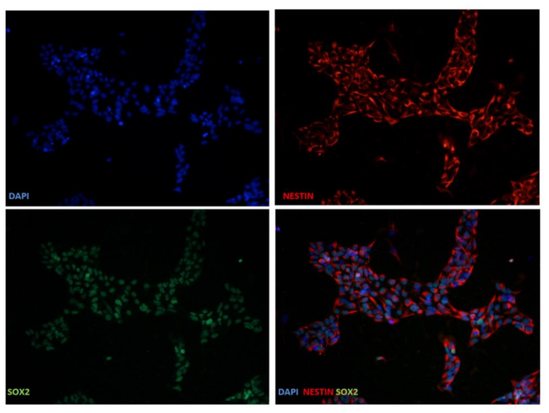

Figure 2. Immunocytochemical staining (ICC) of iNS cells obtained from normal induced pluripotent stem cells (iPSC). iNSC were analyzed for the expression of lineage-specific markers - SOX2 and Nestin.

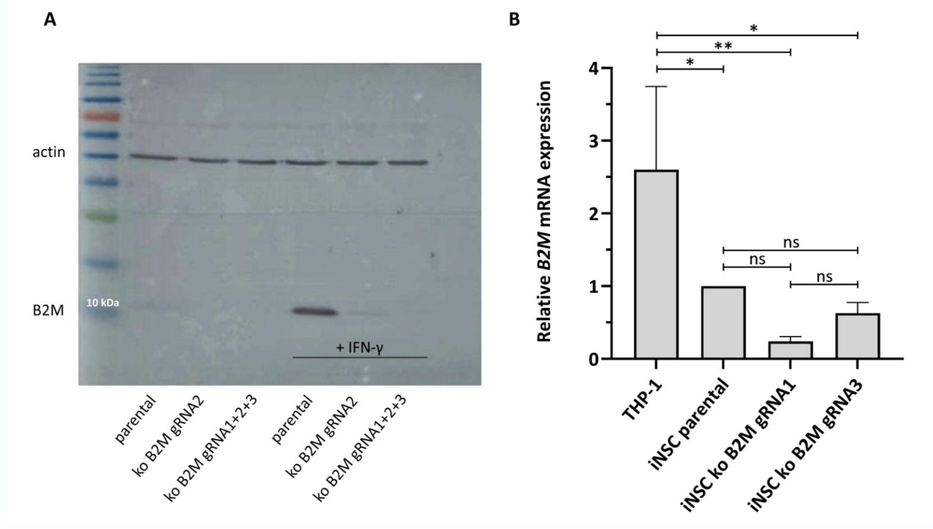

Figure 3. Results of Western Blot analysis (A) and RT-PCR (B) conducted to verify *B2M* knockout in iNS cells. (A) In the case of the first three samples, interferon (IFN-γ) was not added, while in the remaining samples, it was added 24 hours before lysis at a concentration of 500 U/mL to induce B2M expression. (B) Both transduced cells and parental cells were lysed and RNA was isolated and used in real-time PCR. Positive control (with confirmed B2M expression) was provided by cDNA obtained from parental iNS cells. The expression values of the B2M gene for each tested cell line were normalized to the internal control, which was the *TBP* gene. **; *, p<0.05; ns, not significant.

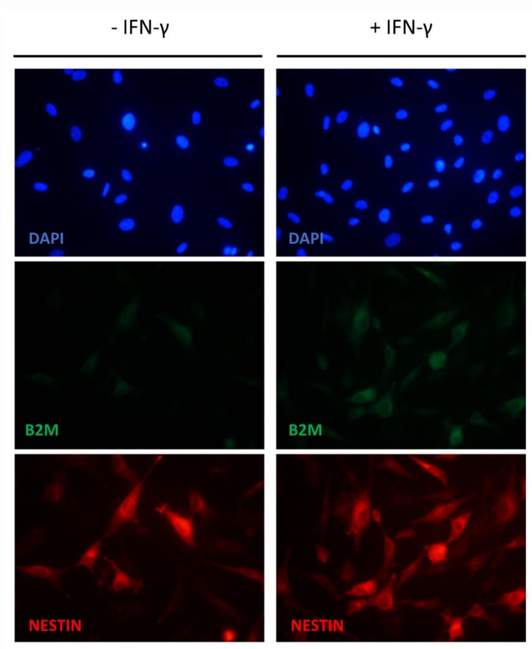

Figure 4. Immunocytochemical staining (ICC) result in iNS cells. To confirm the expression of the B2M gene in iNS cells, immunocytochemical staining was performed using anti-B2M antibody (green signal). At the same time, B2M expression was induced by adding IFN-γ (500 U/mL) for 24 hours..

References

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